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#### ***published in***

Journal of Bacteriology  
2002

#### ***DOI (link to publisher)***

[10.1128/JB.184.9.2557-2560.2002](https://doi.org/10.1128/JB.184.9.2557-2560.2002)

#### ***document version***

Publisher's PDF, also known as Version of record

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#### ***citation for published version (APA)***

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## Nitrite Reductase of *Nitrosomonas europaea* Is Not Essential for Production of Gaseous Nitrogen Oxides and Confers Tolerance to Nitrite

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Received 27 November 2001/Accepted 1 February 2002

**A gene that encodes a periplasmic copper-type nitrite reductase (NirK) was identified in *Nitrosomonas europaea*. Disruption of this gene resulted in the disappearance of Nir activity in cell extracts. The nitrite tolerance of NirK-deficient cells was lower than that of wild-type cells. Unexpectedly, NirK-deficient cells still produced nitric oxide (NO) and nitrous oxide (N<sub>2</sub>O), the latter in greater amounts than that of wild-type cells. This demonstrates that NirK is not essential for the production of NO and N<sub>2</sub>O by *N. europaea*. Inactivation of the putative *fir* gene showed that Fnr is not essential for the expression of *nirK*.**

Oxidation of ammonia (NH<sub>3</sub>) to nitrite (NO<sub>2</sub><sup>−</sup>) is the first step in the nitrification process, which plays an essential role in the global nitrogen cycle. Nitrification involves the oxidation of NH<sub>3</sub>, via NO<sub>2</sub><sup>−</sup>, to nitrate (NO<sub>3</sub><sup>−</sup>) by the combined actions of NH<sub>3</sub>- and NO<sub>2</sub><sup>−</sup>-oxidizing bacteria. The NH<sub>3</sub>-oxidizing bacteria are lithoautotrophs that harvest their vital free energy in the oxidation of NH<sub>3</sub> to NO<sub>2</sub><sup>−</sup>. This process is carried out by the sequential actions of membrane-bound ammonia monooxygenase, which oxidizes NH<sub>3</sub> to hydroxylamine (NH<sub>2</sub>OH), and periplasmic hydroxylamine oxidoreductase (HAO), which oxidizes NH<sub>2</sub>OH to NO<sub>2</sub><sup>−</sup> (24). NH<sub>3</sub>-oxidizing bacteria utilize molecular oxygen both for the formation of NH<sub>2</sub>OH from NH<sub>3</sub> and as the terminal acceptor of electrons from the respiratory chain (24). Alternatively, NO<sub>2</sub><sup>−</sup> and nitric oxide (NO) may also be used as terminal electron acceptors via a putative denitrification pathway. Homologues of genes encoding the denitrification enzymes nitrite reductase (Nir) and nitric oxide reductase (Nor) are present in the genome of the NH<sub>3</sub>-oxidizing bacterium *N. europaea* (22).

NH<sub>3</sub>-oxidizing bacteria produce NO, nitrous oxide, and, in some cases, N<sub>2</sub> during growth on NH<sub>3</sub>. The mechanisms that underlie the production of these nitrogenous gases include the dissimilatory reduction of NO<sub>2</sub><sup>−</sup> (15–17). An enzyme with Nir activity has been isolated from *N. europaea* (3, 6, 12, 13, 18). The ability of *N. europaea* to use NO<sub>2</sub><sup>−</sup> as an alternative electron acceptor suggests that the pathway may act as an alternative mode of respiration, as in the “true” denitrifying bacteria (1, 19). Alternatively, the denitrifying enzymes may serve to protect NH<sub>3</sub>-oxidizing bacteria from the negative effects of NO<sub>2</sub><sup>−</sup> produced during growth (16, 20). On the other hand, the nitrification pathway may also be involved in the production of NO and N<sub>2</sub>O by NH<sub>3</sub>-oxidizing bacteria. HAO of *N. europaea*

has been shown to produce NO and N<sub>2</sub>O during the oxidation of NH<sub>2</sub>OH in vitro (6, 8). Whether HAO also contributes to the production of these gases in vivo is unclear.

We disrupted the putative *nirK* gene of *N. europaea* to learn whether it encodes a functional Nir and to study the effects of mutagenesis of this gene on (i) the production of NO and N<sub>2</sub>O and (ii) the tolerance of the cells toward NO<sub>2</sub><sup>−</sup>.

**Analysis of the *nirK* gene and adjacent loci.** Preliminary sequence data were obtained from The DOE Joint Genome Institute (JGI) ([http://www.jgi.doe.gov/tempweb/JGI\\_microbial/html/index.html](http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html)). An open reading frame (ORF) with homology to genes encoding copper-type Nir enzymes is present in *N. europaea*. This ORF, which we have designated *nirK*, is 930 bp in length and translates into a polypeptide of 309 amino acid residues. An alignment revealed that NirK of *N. europaea* is significantly shorter than other characterized Cu-type Nir proteins (about 50 N-terminal residues). The closest relative of the NirK of *N. europaea* characterized thus far is the outer membrane copper-type Nir of *Neisseria gonorrhoeae*, the precursor of which contains a prokaryotic membrane lipoprotein attachment site (11). However, analysis of the N terminus of NirK of *N. europaea* with the SignalP algorithm (<http://www.cbs.dtu.dk/services/SignalP/>) predicted the presence of a periplasmic target sequence, suggesting that this protein resides in the periplasm.

The *nirK* gene is clustered with three other ORFs in the genome of *N. europaea* (Fig. 1). ORF 1 translates into a protein of which the predicted N terminus of the mature form matched that of a previously isolated soluble blue copper oxidase that we sequenced in this study (EKREFDLSIEDTRIVLVGKRDFHTFAFNGQVPAPLIHVM) (3). ORFs 2 and 3 encode periplasmic c-heme-containing polypeptides that have been characterized by Whittaker and coworkers (22).

**Biochemical characterization and complementation of a NirK-deficient mutant.** *N. europaea* strain ATCC 19178 and NirK-deficient mutant XLnt (ATCC 19178 derivative; *nirK*::pNIRsu [this study]) were grown in liquid medium as

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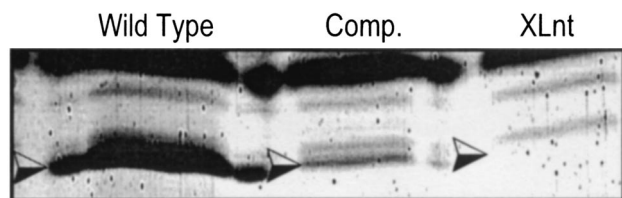


FIG. 2. SDS-PAGE of periplasmic protein fractions. WT, wild type; Comp., complemented NirK deficient; XLnt, NirK deficient. The arrowheads indicate the position of the NirK protein band, which is not present in the extract from NirK-deficient cells.

on the same order of magnitude, around  $5 \mu\text{M}$ . NO production rates were also monitored by using oxygenated hemoglobin, which reacts rapidly with NO, resulting in a shift in the UV-visible light absorption spectrum of hemoglobin (14). When headspace gas from either wild-type or NirK-deficient cell cultures was led through an oxygenated hemoglobin solution, this shift occurred at similar rates, illustrating that the NO production rates of the two strains were similar.

The  $\text{N}_2\text{O}$  concentration in the headspace of batch cultures of wild-type and NirK-deficient cells was measured with a gas chromatograph (Table 1). Remarkably, the concentration of  $\text{N}_2\text{O}$  in the headspace of the NirK-deficient cultures was approximately threefold higher than that in the headspace of the wild-type cell cultures. Next, equal amounts of exponentially growing cells in liquid cultures were transferred to new flasks (50 ml) that were sealed with rubber stoppers and incubated at  $30^\circ\text{C}$  for 50 min with continuous shaking (175 rpm). Subsequently, samples were taken from the headspace at 10-min intervals for measurement of the  $\text{N}_2\text{O}$  concentration. The NirK-deficient cells produced  $\text{N}_2\text{O}$  at a rate that was approximately four times greater than that of the wild-type cells (Table 1). The  $\text{NH}_3$ -dependent oxygen uptake rates of the cultures used in this experiment were found to be similar, indicating that the respiratory potentials of the wild-type and NirK-deficient cells were comparable (Table 1).

**Growth characteristics of the NirK-deficient mutant.** The growth characteristics of wild-type and NirK-deficient cells of

*N. europaea* were determined by measurement of the time-dependent increase in turbidity of aerobic batch cultures (Fig. 3a and b). The cultures (150 ml) were incubated in 500-ml flasks (with semiloose caps to facilitate gas exchange) on an angled ( $70^\circ$ ) rotary shaker (175 rpm) at  $30^\circ\text{C}$  in the dark. The specific growth rates of wild-type and NirK-deficient cells were similar, approximately  $0.1 \text{ h}^{-1}$ . The NirK-deficient cells reached a maximal biomass concentration that was approximately 90% of that of the wild-type cells.

**Effect of  $\text{NO}_2^-$  on growth.** Involvement of NirK in the tolerance of cells of *N. europaea* toward  $\text{NO}_2^-$ , which can be toxic to the cells (20), was assessed by examination of the growth characteristics of wild-type and NirK-deficient cells in a series of batch cultures to which increasing amounts of  $\text{NO}_2^-$  were added at the start of culturing (Fig. 3a and b). Culturing conditions were as described above. The addition of increasing amounts of  $\text{NO}_2^-$  had increasing negative effects on the specific growth rate and maximal biomass concentration of cultures of wild-type and NirK-deficient cells. However, these effects were more profound for the NirK-deficient cells than for the wild-type cells at each given  $\text{NO}_2^-$  concentration. At the highest concentration tested (100 mM), wild-type cells were still capable of growth while NirK-deficient cells were not. This demonstrates that NirK confers tolerance to  $\text{NO}_2^-$ . The concentration of  $\text{NO}_2^-$  in the growth medium was measured during growth of the cultures to which 0 and 10 mM  $\text{NO}_2^-$  had been added (Fig. 3c). Under both conditions, the concentration of  $\text{NO}_2^-$  in the cultures of wild-type cells exceeded those in the medium of the NirK-deficient cells at all measured points. It is likely that this was due to the increased negative effects of  $\text{NO}_2^-$  on the growth of NirK-deficient cells. This observation indicates that the mechanism by which NirK reduces the negative effects of  $\text{NO}_2^-$  on growth does not involve lowering of the amount of extracellular  $\text{NO}_2^-$  to which the cells were exposed.

**Role of Fnr in regulation of the *nirK* gene.** The genome of *N. europaea* contains a gene that encodes a protein with a high degree of homology to transcription activators that belong to the Fnr/Crp family. Fnr of *N. europaea* contains the cysteine

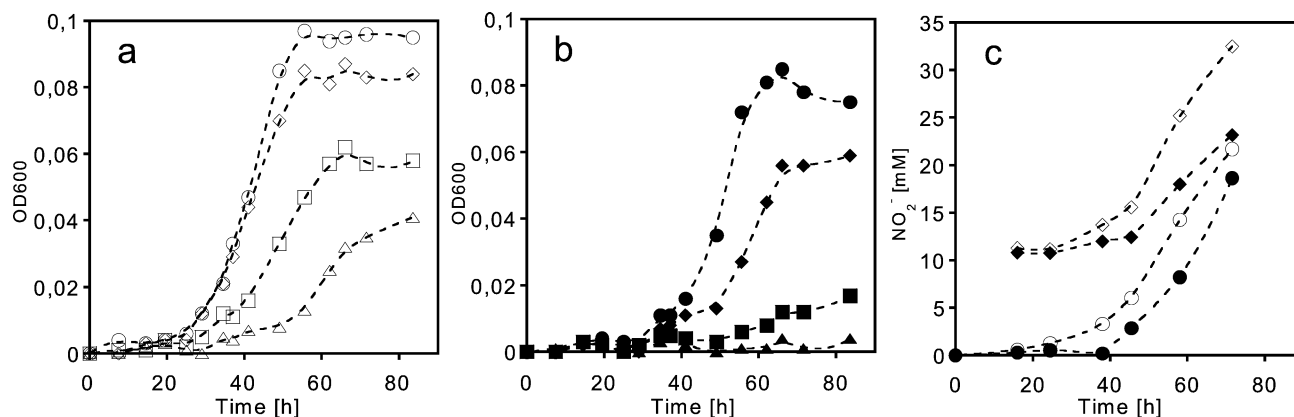


FIG. 3. Growth curves of wild-type (a) and NirK-deficient (b) cells of *N. europaea* incubated in aerobic batch cultures with 0 mM (circles), 10 mM (diamonds), 50 mM (squares), and 100 mM (triangles)  $\text{NaNO}_2$  present in the growth medium at the start of the incubation. OD600, optical density at 600 nm. (c) Concentrations of  $\text{NO}_2^-$ , measured in a comparable experiment, in the growth medium of batch cultures of wild-type (open symbols) and NirK-deficient (filled symbols) cells of *N. europaea* with 0 mM (circles) and 10 mM (diamonds)  $\text{NaNO}_2$  present in the medium at the start of the incubation. All experiments were done in triplicate, and representative curves are shown.



residues that align with those in *E. coli* Fnr and which are involved in the ligation of the oxygen-sensing [4Fe-4S] cluster. Fnr is involved in control of the expression of the denitrification enzymes in various bacteria (10, 25). For this reason, we constructed and analyzed an Fnr-deficient strain. NirK was still expressed in this mutant, which demonstrates that the putative Fnr of *N. europaea* is not essential for transcription of the *nirK* gene.

**Conclusions.** *N. europaea* possesses a gene that encodes a functional copper-type NirK that resides in the periplasmic space. Based on the findings presented here, we conclude that NirK is essential for the production of neither NO nor N<sub>2</sub>O in *N. europaea*. The absence of NirK resulted in a decreased tolerance to NO<sub>2</sub><sup>-</sup>, indicating that it may serve to protect the cell from the negative effects of this product of NH<sub>3</sub> oxidation. A model of the respiratory network of *N. europaea* that encompasses a linear denitrification pathway comprising NirK and Nor predicts that absence of NirK would render the system incapable of the production of NO and N<sub>2</sub>O. Clearly, *N. europaea* has an alternative pathway for the production of these gases. Since HAO has been demonstrated to produce NO and N<sub>2</sub>O in vitro, this key enzyme of the nitrifying pathway is also likely to be involved in this alternative pathway in vivo (6, 7).

The finding of *nirK* gene homologues in oceanic NH<sub>3</sub>-oxidizing bacteria illustrates that NirK is not unique to *N. europaea* but appears to be widespread among the group of NH<sub>3</sub>-oxidizing bacteria (2).

This work was financially supported by The Netherlands Organization for Scientific Research (NWO).

We thank T. Iizumi for the conjugation protocol; N. Saunders, W. N. M. Reijnders, and B. van Schooten for technical assistance; I. Schmidt for NO<sub>x</sub> analyses; and R. van der Schors for the Q-TOF-MS analyses. All preliminary sequence data were obtained from The DOE Joint Genome Institute (JGI) ([http://www.jgi.doe.gov/tempweb/JGI\\_microbial/html/index.html](http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html)).

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